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Assays for Acetaldehyde-Derived Adducts in Blood Proteins Based on Antibodies Against Acetaldehyde/Lipoprotein Condensates

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Background: Acetaldehyde-derived protein condensates (adducts) have been suggested as promising biological markers of alcohol abuse because they represent actual metabolites of ethanol. However, the detection of such condensates in vivo has been hampered by a lack of sensitive and specific methods.

Methods: To develop new approaches for the detection of acetaldehyde adducts, we have raised antibodies against condensates with acetaldehyde and lipoproteins, which have previously been shown to be readily modified by acetaldehyde in vitro. The characteristics of these antibodies were compared with those raised against bovine serum albumin/acetaldehyde adduct and against other types of lipoprotein modifications, as induced by malondialdehyde, oxidation, and acetylation.

Results: The antibodies raised against low-density lipoprotein (LDL)/acetaldehyde, very low density lipoprotein (VLDL)/acetaldehyde, and bovine serum albumin/acetaldehyde all reacted with protein adducts generated at physiologically relevant concentrations of acetaldehyde in vitro, whereas the antibodies raised against malondialdehyde/LDL, oxidized LDL, or acetylated LDL were not found to cross-react with the acetaldehyde-derived adducts. In assays for acetaldehyde adducts from erythrocyte and serum proteins of patients with excessive ethanol consumption (n = 32) and healthy control individuals (n = 22), the antibody prepared against the acetaldehyde/VLDL condensate was found to provide the most effective detection of acetaldehyde adducts in vivo.

Conclusions: Current data indicate that acetaldehyde generates immunogenic adducts with lipoproteins in vivo. Antibodies raised against the VLDL/acetaldehyde may provide a basis for new diagnostic assays to examine excessive alcohol consumption.

Key Words: Ethanol Metabolism, Lipoproteins, Alcohol Markers.

GENERATION OF PROTEIN adducts with acetaldehyde, the first metabolite of ethanol, has been previously well established in vitro. Acetaldehyde is known to bind to reactive amino acid residues in several target proteins (Israel et al., 1986; Stevens et al., 1981; Tuma and Sorrell, 1995; Wehr et al., 1993; Zhu et al., 1996). Under reducing conditions, especially lysine-rich sites of the proteins seem to become modified at physiologically relevant concentrations of acetaldehyde (Braun et al., 1995; Jennett et al., 1989; Tuma et al., 1987). Studies with erythrocyte proteins have also indicated that stable cyclic imidazolidinone derivatives can be formed between acetaldehyde and the free α-amino groups of the amino-terminal valine of hemoglobin (Braun et al., 1997; Fowles et al., 1996; San George and Hoberman, 1986).

In theory, acetaldehyde/protein condensates would be ideal biological markers of alcohol consumption because they should represent actual metabolites of ethanol as integral parts of the analyte. However, no routine applications for adduct measurements have so far been developed. Previous reports on assays for acetaldehyde adducts have used antibodies recognizing reduced epitopes of acetaldehyde condensates with bovine serum albumin (BSA; Niemelä and Israel, 1992) or keyhole limpet hemocyanin (Lin et al., 1993). Although such assays have revealed increased levels of acetaldehyde adducts in erythrocyte proteins of alcoholic patients, the specificity and sensitivity of the assays have not been sufficient for routine diagnostic use.

On the basis of earlier observations indicating that lipoproteins of alcoholics are readily modified by acetaldehyde in vivo (Lin et al., 1995; Wehr et al., 1993), we have initiated experiments to induce antibodies against various types of lipoprotein modifications. It seems that the use of such antibodies may improve the detection of acetaldehyde adducts as markers of problem drinking.

MATERIALS AND METHODS

Patients and Control Subjects

For patient assays, ethylenediaminetetraacetic acid (EDTA) blood samples and serum samples were collected from 32 patients hospitalized

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Table 1. Clinical and Biochemical Characteristics of the Study Population

Group	Men/women	Age (yr)	Hb (g/l)	MCV (fl)	GT(u/l)	ALT(u/l)	AST(u/l)	ALP(u/l)	BIL(μmol/1)	ALB(g/l)
Alcoholics (n = 32)	30/2	45 ± 8	151 ± 9	97 ± 5	168 ± 264	71 ± 79	75 ± 92	153 ± 40	12 ± 7	39 ± 3
Controls (n = 22)	11/11	44 ± 8	144 ± 10	93 ± 3	26 ± 11	28 ± 17	32 ± 33	115 ± 30	13 ± 6	42 ± 3

The values are expressed as mean ± SD.
Hb, hemoglobin; MCV, mean corpuscular volume; GT, serum γ glutamyltransferase; ALT, serum alanine aminotransferase; AST, serum aspartate aminotransferase; ALP, serum alkaline phosphatase; BIL, serum bilirubin; ALB, serum albumin.

for detoxification (30 men and 2 women; mean age, 46 years; range, 32–59 years). They were treated and observed at the Department of Psychiatry, EP Central Hospital, Seinäjoki, Finland. All patients had a history of heavy drinking consisting of more than a mean of 60 g of ethanol per day, either continuously or during repeated inebriations. The time of abstinence before sampling was from 0 to 7 days, such that blood samples from 22 patients were taken at the day of admission, from 5 patients from 1 to 3 days, and from 5 patients from 4 to 7 days after admission. Controls were 22 healthy individuals (11 men and 11 women; mean age, 45 years; range, 25–59 years) who were either teetotalers or social drinkers consuming less than 20 g of ethanol per day, with a maximum weekly consumption of 60 g. None of them had consumed alcoholic beverages during the period of 1 week before sampling. The relevant clinical and biochemical characteristics of the study population are summarized in Table 1. Erythrocyte hemolysates from the EDTA-anticoagulated blood and serum samples were prepared from all study subjects and kept frozen at −70°C until use. The study was approved by the Institutional Ethical Committee, and it was performed according to the provisions of the Declaration of Helsinki.

Preparation of Lipoprotein Modifications

For the preparation of the various derivatized lipoproteins, blood samples were first collected from a healthy nondrinking control subject. Low-density lipoprotein (LDL) and very low density lipoprotein (VLDL) fractions were prepared by ultracentrifugation.

Acetaldehyde Modifications. Previously established methods were followed to prepare acetaldehyde condensates with lipoproteins (Niemelä et al., 1991, 1994), erythrocyte proteins, and BSA (Israel et al., 1986; Niemelä et al., 1991, 1994). In essence, the proteins were incubated in phosphate-buffered saline (PBS) solution containing various concentrations of acetaldehyde (range, 0–10 mM). Sodium cyanoborohydride (10 mM) (Sigma Chemical Co., St. Louis, MO) was used to stabilize the condensates. The acetaldehyde solutions in buffer were prepared by appropriate dilutions of stock solutions of 1,2-^{14}C-acetaldehyde (185 MBq/mmol, NEC-374, Lot 2212-171, New England Nuclear, Boston, MA) with unlabeled acetaldehyde.

Modification of lipoproteins with malondialdehyde (MDA) was performed by incubating lipoproteins for 3 hr at 37°C with 0.5 M MDA (Haberland et al., 1982; Niemelä et al., 1994; Palinski et al., 1990). MDA was freshly generated from MDA bis dimethylacetal by acid hydrolysis.

Acetylated Proteins. Human LDL was acetylated according to the procedure of Basu et al. (1976). In a typical preparation, 2 ml of 0.15 M NaCl containing 2 mg of LDL protein was added to 2 ml of a saturated solution of sodium acetate with continuous stirring in an ice-water bath. Next, acetic anhydride was added in multiple small aliquots over 1 hr with continuous stirring. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of the protein used, the mixture was stirred for an additional 30 min without further additions. The reaction solution was then dialyzed for 24 hr at 4°C against PBS containing 10 μM EDTA, pH 7.4.

Oxidized Proteins. Human LDL was oxidized by exposure of 200 μg/ml apolipoprotein to copper sulfate (5 μM free copper concentration) in PBS at 37°C for 20 hr.

Immunizations and Antibody Titer Determinations

Antisera against acetaldehyde-modified lipoprotein fractions, acetaldehyde-modified albumin, MDA modification, acetylated proteins, and oxidized proteins were generated by immunizing rabbits with homologous protein modifications that had been freshly prepared and stored at 4°C until used for immunizations. The primary immunization consisted of intradermal injections of 500 μg (BSA/acetaldehyde, MDA-modified lipoprotein, and acetylated and oxidized lipoproteins) or 250 μg (LDL/acetaldehyde) or 125 μg (VLDL/acetaldehyde) of the modified proteins suspended in PBS and Freund's complete adjuvant (Difco Laboratories, Detroit, MI; 1:1 ratio). Booster injections of 250 μg of the antigen in Freund's incomplete adjuvant were given at 3- to 4-week intervals.

For antibody titer determinations, microtiter plate wells were coated with different freshly prepared antigen preparations, with which the various antisera were allowed to react. Antigen concentrations of 1 to 5 μg per well, representing different degrees of modification, were used. The antigen-containing solutions were allowed to react with various dilutions of antisera for 1 hr at +37°C. The second antibody was goat anti-rabbit immunoglobulin labeled with alkaline phosphatase. *p*-Nitrophenylphosphate solution was added for color reaction. The intensities of the reactions were measured by an Anthos (Anthos Labtec Instruments, Salzburg, Austria) HtII microplate reader.

Immunoassays for Acetaldehyde Adducts From Patient Samples

For assays from erythrocyte proteins, hemolysates were prepared from 32 alcohol-consuming patients and from 22 healthy controls. Microtiter plates were first coated with ammonium sulphate-precipitated, affinity-purified antibodies (1:50 dilution). The reaction was allowed to proceed overnight at +4°C. After blocking with 0.2% gelatin/PBS, patient hemolysate samples were added (120 μg protein per well). The plates were incubated at +37°C for 1.5 hr and washed with PBS containing 0.04% Tween-20 (ICI Americas, Inc., Wilmington, DE). Subsequently, sheep anti-human hemoglobin antiserum (Biogenesis, Poole, Dorset, UK) was added (dilution 1:1500), and the reaction was allowed to proceed for 30 min at +37°C. After extensive washes, alkaline phosphatase–linked rabbit anti-sheep immunoglobulin G heavy + light chains of the immunoglobulin molecule (H+L) (Zymed Laboratories, South San Francisco, CA) was added and allowed to react for 1 hr at +37°C to precipitate antigen-antibody complexes. An Alkaline Phosphatase Substrate Kit from Bio-Rad (Hercules, CA) was used for color reaction.

Similarly, for assays of serum proteins, microtiter plates were first coated with the purified antibodies. After the addition of patient serum samples (50 μg protein per well), the plates were incubated at +37°C for 1.5 hr and washed with PBS-Tween. Subsequently, goat anti-human serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) antiserum was added (dilution 1:10,000) and allowed to react for 30 min at +37°C. After extensive washes, alkaline phosphatase–conjugated affinity-purified rabbit anti-goat immunoglobulin G (H+L) (Jackson Immunoresearch Laboratories, Inc.), dilution 1:5000 was added and allowed to react for 1 hr at +37°C to precipitate antigen-antibody complexes.

Other Methods

Blood chemistries, serum enzyme, bilirubin, and albumin concentrations were measured with standard clinical chemical methods.

Statistical Methods

The comparison between the alcoholic and nonalcoholic groups was performed with Student's *t* test. Logarithmic transformation was used for the variables that followed unsymmetrical distribution (measurements

Table 2. The Reactions of the Antibodies Raised Against Different Types of Lipoprotein Modifications and Acetaldehyde-Modified Erythrocyte Proteins Prepared at Different Concentrations of Acetaldehyde

	Acetaldehyde/protein condensates prepared at different concentrations of acetaldehyde				
Antibody against	10 mM	1 mM	100 μM	20 μM	5 μM
Acetaldehyde (100 μM)/LDL	+++	++ (+)	++	+ (+)	+
Acetaldehyde (100 μM)/VLDL	++ (+)	++	++	+ (+)	+ (+)
BSA (1 mM)/acetaldehyde	+++	+++	++	+	(+)
MDA/LDL	—	—	—	—	—
Oxidized LDL	—	—	—	—	—
Acetylated LDL	—	—	—	—	—

The intensities of the immunoreactions are given as means of relative intensities (OD_{405}), as follows: (+), 0–0.2; +, 0.2–0.4; +(+), 0.4–0.6; ++, 0.6–0.8; ++(+), 0.8–1.0; +++, more than 1.0. LDL, low-density lipoprotein; VLDL, very low density lipoprotein; MDA, malondialdehyde; BSA, bovine serum albumin.

from hemolysates). The correlations were calculated with Pearson's product moment correlation coefficients.

RESULTS

Immunization of rabbits with the acetaldehyde-derivatized LDL, VLDL, and BSA resulted in the production of antibodies, which recognized acetaldehyde adducts prepared at physiologically relevant concentrations of acetaldehyde (Table 2), whereas this was not the case with epitopes resulting from lipoprotein oxidation, acetylation, or MDA modification. Similarly, the antibodies raised against MDA/lipoprotein and acetylated and oxidized lipoproteins were not found to react with the acetaldehyde-derived condensates (Table 2). The antiserum raised against acetaldehyde/VLDL adduct was found to show the highest affinity toward the acetaldehyde condensates prepared with low (5–20 μM) concentrations of acetaldehyde.

The antibodies were subsequently compared in immunoassays for acetaldehyde adducts from erythrocyte proteins and serum samples of individuals with various degrees of alcohol consumption. Figure 1 demonstrates the data on the assays from erythrocyte proteins of 32 problem drinkers and of 22 control individuals. In these specimens, the acetaldehyde adduct levels, as analyzed with the anti-VLDL/acetaldehyde adduct antibody, were significantly higher in the alcohol-consuming patients than in the control patients (Fig. 1A). The anti-LDL adduct (Fig. 1B) and anti-BSA adduct (Fig. 1C) antibodies also revealed higher values in the alcoholic samples, but the differences between the alcohol abusers and control subjects did not reach significance.

Figure 2 demonstrates the results of the assays for acetaldehyde adducts from serum samples. Similarly, measurements with the anti-VLDL adduct antibody revealed significantly higher concentrations of immunoreactive adducts in the serum of the alcoholic patients (Fig. 2A). The anti-LDL adduct (Fig. 2B) and the anti-BSA adduct (Fig. 2C) antibodies also separated between the alcoholic samples and nonalcoholic samples, but the differences were less striking.

In 22 alcoholic patients, the blood sample was taken at the day of admission for detoxification, whereas there were 5 patients in whom the sample was taken after 1 to 3 days of abstinence and another 5 in whom the sample was taken 4 to 7 days after admission. Table 3 demonstrates the values obtained from the patients, as classified according to the number

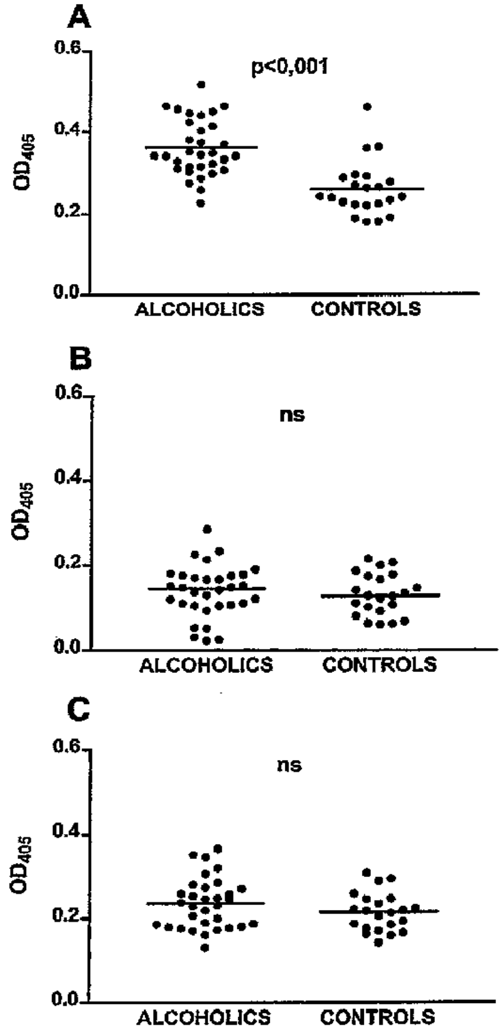

Fig. 1. The results of the enzyme-linked immunosorbent assays for the acetaldehyde adducts of erythrocyte proteins from alcoholics admitted for detoxification and from nondrinking controls. The immunoassay plates were coated with antibodies against acetaldehyde/VLDL (A), acetaldehyde/LDL (B), and acetaldehyde/BSA (C), with which the hemolysates were allowed to react. Antigen-antibody complexes were detected with sheep anti-human hemoglobin and alkaline phosphatase-linked rabbit anti-sheep immunoglobulin G. The antibody raised against the VLDL/acetaldehyde condensate was found to be most effective in separating the alcoholic and nonalcoholic population; ns, not significant. OD_{405} = optical density read at 405 nm.

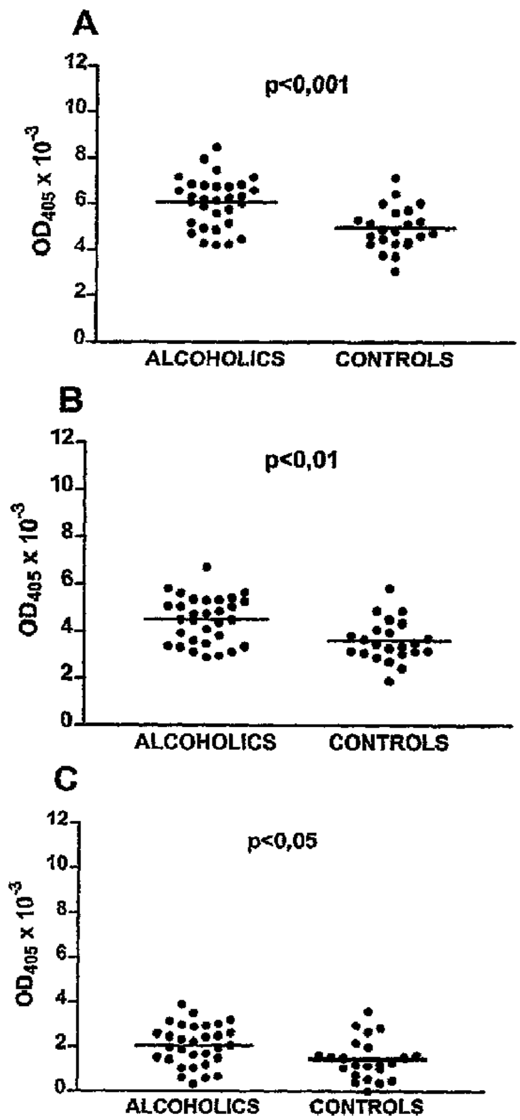

Fig. 2. The results of the assays for the acetaldehyde adducts from serum proteins of alcoholics admitted for detoxification and in nondrinking controls. The immunoassay plates were coated with antibodies against acetaldehyde/VLDL (A), acetaldehyde/LDL (B), and acetaldehyde/BSA (C), with which the serum proteins (50 μg per well) were allowed to react. Antigen-antibody complexes were detected with goat anti-human serum and alkaline phosphatase-linked rabbit anti-goat immunoglobulin G. The results are expressed as optical density read at 405 nm (OD_{405}) units per protein. The antibody raised against the VLDL/acetaldehyde showed the most effective separation between the alcoholic and nonalcoholic populations.

of days of abstinence. Whereas the erythrocyte adducts were found to remain relatively constant, the serum adducts showed decreasing adduct levels in these comparisons (Table 3).

DISCUSSION

These data report on new approaches for the study of acetaldehyde adducts in biological samples on the basis of antibodies raised against acetaldehyde condensates with VLDL. The ability of acetaldehyde to bind to lipoproteins has been previously well documented (Savolainen et al., 1987; Steinbrecher et al., 1984; Wehr et al., 1993). Previous evidence indicating that such binding also seems to occur in vivo in alcohol abusers, create immunologic responses (Wehr et al., 1993), activate apolipoprotein E synthesis in macrophages, and promote atherogenesis (Lin et al., 1995) led us to investigate whether assays based on the immunogenic epitopes of acetaldehyde/lipoprotein condensates could also prove to be useful in the detection of problem drinking.

Table 3. Results of the Immunologic Assays From Erythrocyte and Serum Proteins With the Various Antibodies in Patients Classified According to the Time of Abstinence Before Sampling

		Antibody against		
Days of abstinence	*n*	VLDL/Ach	LDL/Ach	BSA/Ach
Erythrocyte proteins				
0	22	0.368 ± 0.063	0.150 ± 0.063	0.247 ± 0.046
1–3	5	0.347 ± 0.089	0.160 ± 0.061	0.259 ± 0.068
4–7	5	0.362 ± 0.088	0.176 ± 0.043	0.297 ± 0.070
Serum proteins				
0	21	6.35 ± 1.07	4.76 ± 0.95	2.32 ± 0.88
1–3	6	5.80 ± 1.10	4.21 ± 0.95	1.86 ± 0.56
4–7	5	5.20 ± 0.87	3.64 ± 0.72	1.18 ± 0.94

The values are means ± SD in units described in "Materials and Methods." Ach, acetaldehyde.

Several previous trials on acetaldehyde adduct measurements have been performed with immunologic ion-exchange chromatography and HPLC techniques. For immunologic measurements, antibodies against acetaldehyde/protein condensates have been previously generated by using BSA (Niemelä and Israel, 1992) or keyhole limpet hemocyanin (Lin et al., 1993) as carrier proteins. Previous studies have established that generation of aldehyde/protein adducts readily induces immunologic responses (Israel et al., 1986; Viitala et al., 1997; Worrall et al., 1991). These data indicate that the acetaldehyde-derived adducts possibly enriched in large-sized VLDL apolipoprotein B/protein modifications may be particularly strong immunogens. Ethylation of apolipoprotein B/lysine residues in VLDL also seems to share the immunologic determinants of the adducts generated with proteins in vivo. In the liver, where modification of VLDL is expected to take place upon ethanol consumption and generation of acetaldehyde, the VLDL particles are homogeneous large-sized particles, which may contain modified proteins at a greater concentration than the smaller particles found in circulation.

Immunologic measurements of adducts from proteins in circulation have been stimulated by the findings that the antibodies generated against the aldehyde-derived epitopes recognize specific protein adducts independently of the nature of the carrier protein (Israel et al., 1986; Steinbrecher et al., 1984). Previous studies on acetaldehyde/protein adducts have reported high adduct concentrations in erythrocytes of chronic alcoholics (Gross et al., 1992; Hurme et al., 1998; Lin et al., 1993; Niemelä and Israel, 1992; Sil-

lanaukee et al., 1992). Some previous studies with various relatively cumbersome or indirect techniques have also reported evidence for the existence of protein adducts in plasma proteins (Chen et al., 1995; Lin et al., 1990; Peterson and Polizzi, 1987; Wickramasinghe et al., 1986). Acetaldehyde-modified epitopes have also been found from the surface of hepatocytes by flow cytometry (Lin et al., 1992; Trudell et al., 1990) and from liver tissue from patients with alcoholic liver disease (Holstege et al., 1994; Niemelä et al., 1991, 1994). However, studies by immunologic methods have usually failed to detect acetaldehyde-modified proteins from serum specimens. In this work, the VLDL adduct antibodies were, however, found to react with adducts in both the erythrocyte and serum compartments.

Current data further indicate that although MDA, an aldehydic product of lipid peroxidation, also forms Schiff's base adducts with proteins, the immunogenic epitopes generated by acetaldehyde and MDA do not significantly cross-react with each other. Acetaldehyde and MDA adducts may, however, coexist in alcoholics (French et al., 1993; Niemelä et al., 1995; Viitala et al., 2000; Xu et al., 1998), especially when ethanol consumption is combined with a high-fat diet or iron overload (Niemelä et al., 1998; Tsukamoto et al., 1995). MDA is generated during nonenzymatic lipid peroxidation of unsaturated fatty acids, in lipid peroxidation during phagocytosis by monocytes, and in arachidonic acid catabolism in thrombocytes (Esterbauer et al., 1991; Palinski et al., 1990). Oxidative modification of proteins has been previously demonstrated from arterial vessel walls of atherosclerotic lesions (Haberland et al., 1988; Palinski et al., 1989; Steinberg et al., 1989). As yet, no studies are, however, available on the existence of circulating MDA-modified epitopes in erythrocytes or in serum proteins from alcoholics. Antibodies against oxidative modifications may, however, also be found from the serum of alcoholics with severe liver disease (Viitala et al., 2000).

Taken together, these data indicate that immunogenic acetaldehyde/lipoprotein adducts are formed in vivo and may be used in the production of antibodies in laboratory animals. Such antibodies may prove to be of value in the detection of protein adducts formed in vivo as a result of alcohol intake.

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